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Dear FYI Coordinator:

In accordance with API's policy of providing the federal government with copies of research designed to determine whether any chemical substance or mixture manufactured, processed or distributed by API member companies may cause a risk of injury to health or the environment, we are enclosing a copy of the following draft report:

**(Identification no. FYI not assigned) Pilot Experiment Using CD-1 Mice --
Dermigen Protocol 910828, Draft Final Report.**

Please note that this information is provided in accordance with the full disclosure policy of API and does not constitute a formal submission as required by a test rule.

This document does not contain confidential information. If you have any questions, please communicate with me.

Sincerely,

Robert T. Drew, Ph.D.

DERMIGEN

ON THE ADVANCING EDGE

*Biology, biochemistry and toxicology of the dermis
Consulting and contract research*

Draft Final Report - February 9, 1994

Pilot Experiment Using CD-1 Mice - Dermigen Protocol No. 910828 (9/28/93)

I. Purpose

This experiment was designed: 1) to determine the effect of biopsy (wounding) on the induction of epidermal hyperplasia and epidermal ornithine decarboxylase (ODC) activity in subsequent biopsies; and 2) to establish a biopsy size that would provide adequate material for replicate assays of ODC activity.

II. Methods

A. Experimental animals

Female CD-1 mice (20) were purchased from Charles River Breeding Farms at 5 to 6 weeks-of-age. At 7 to 9 weeks-of-age the backs of the mice were shaved with surgical clippers and housed for 2 days prior to entry onto protocol. Only those mice in the resting phase of the hair growth cycle were utilized.

B. Treatment of mice and collection of skin biopsies

The tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA from LC Services, Woburn, MA), was dissolved in spectral grade acetone and applied to the shaved backs of 10 mice (6.8 nmole in 200 μ L). Ten control mice received applications of 200 μ L acetone. Mice were administered up to 6 applications of TPA or acetone (2 x/wk on Monday and Thursday). At 6, 24, or 48 hr following the fourth or sixth application of TPA, mice were lightly anesthetized with Metophane™. Skin biopsies were taken using 3, 4, or 6 mm sterile disposable punches (Accuderm, Ft. Lauderdale, FL). These punches produced full thickness skin biopsies of very uniform thickness.

After 4 applications of acetone or TPA, the following biopsies were taken from groups of 3 mice, with biopsy centers sited as described in Protocol No. 910828:

6 hr post-final dosing	6 mm
24 hr post-final dosing	3 mm + 6 mm
48 hr post-final dosing	3 mm

The biopsies from 3 mice within a single experimental group were pooled prior to homogenization and preparation of the supernates for assay of ODC activity. In addition, 6 mm biopsies were removed from 1 or 2 mice at 6 hr post-final dosing to determine if ODC could be measured in only 1 or 2 biopsies.

After 6 applications of acetone or TPA, the following biopsies were taken from groups of 3 mice, with biopsy centers sited as described in Protocol No. 910828:

6 hr post-final dosing	4 mm
24 hr post-final dosing	3 mm + 4 mm
48 hr post-final dosing	3 mm

Again, biopsies from 3 mice within a single experimental group were pooled prior to homogenization and preparation of the supernates for assay of ODC activity.

Optimum excision of the skin specimens required that the skin be gently stretched while rotating the punch biopsy.

Thirty minutes prior to biopsy at 48 hr post-final dosing, mice were injected i. p. with bromodeoxyuridine (BrdUrd), 100 µg/gm body weight.

C. Assay of epidermal ODC activity

ODC activity was measured in supernatant fractions of 4 or 6 mm skin biopsies as described by O'Brien et al (1) and Kruszewski and DiGiovanni (2). Briefly, single punch biopsies from each of 3 mice in the same experimental group were pooled and homogenized with a Polytron PT-10/35 homogenizer (Brinkman Instruments, Westbury, NY) in 1.0 mL of 50 mM phosphate buffer (pH 7.2), containing 0.1 mM pyridoxal phosphate, 5 mM dithiothreitol and 0.1 mM EDTA. No problems were experienced in homogenizing the full thickness skin biopsies. The homogenate was centrifuged at 30,000 x g for 30 min. ODC activity in the supernate was determined by measuring the release of $^{14}\text{CO}_2$ from L-[1- ^{14}C] ornithine hydrochloride. Enzyme activity was expressed as nmol CO_2 liberated/hr per mg protein or per surface area (mm^2) of skin.

D. Assessment of epidermal hyperplasia

1. Fixation and processing of 3 mm biopsies

The biopsies were fixed in neutral buffered formalin and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin

(H&E) for epidermal thickness and for immunochemical visualization of BrdUrd, according to procedures specified in Protocol No. 910828.

2. Measurement of epidermal thickness

Measurement of this parameter was accomplished using a calibrated eyepiece and examining the interfollicular epidermis of H&E-stained sections. A minimum of 5 determinations were performed on each slide. In addition, each specimen was evaluated for the presence of necrosis or signs of inflammation.

3. Measurement of labeling (BrdUrd) index

The detection of BrdUrd-labeled epidermal cells was accomplished using a monoclonal antibody against BrdUrd (Becton Dickinson, San Jose, CA) and a biotinylated secondary antibody (Vector). The biotinylated complex was detected with a biotin-avidin-immunoperoxidase kit (Vectastain, Vector). For visualization of the cells, sections were lightly counterstained with hematoxylin. Determination of the percentage of BrdUrd-labeled cells was accomplished by counting a minimum of 300 cells/slide.

III. Results

A. Biopsy size for assay of ODC activity

The Veterinary-in-Charge at the Science Park deemed the wound produced by the 6 mm biopsy to be of sufficient size to require closure, preferably using a wound clip. Although this was done, the procedure involves more manipulation of the skin than desired or deemed appropriate for the experimentation. In view of this, a decision was made to utilize a 4 mm biopsy, which in the opinion of the Veterinarian-in-Charge did not require closure. Since extra mice had been entered onto protocol, it was decided to extend TPA treatments for an additional week and perform ODC assays on 4 mm biopsies for ODC assay. Unpublished data from Dr. DiGiovanni's laboratory and observations of Aldaz et al. (3) indicate that no difference in epidermal hyperplasia is observed between 4 and 6 applications of TPA. All ODC assays reported herein are for mice treated with 6 applications of TPA or acetone.

B. Histopathologic assessment

Histopathologic assessment of 3 mm biopsies revealed no evidence of inflammation that would suggest an effect of a prior biopsy on a subsequent biopsy. TPA-treated skin exhibited no areas of necrosis.

C. TPA-induced effects on epidermal hyperplasia

Table 1 summarizes the effects of TPA on epidermal thickness and labeling (BrdUrd) index of epidermal cells.

D. TPA induction of epidermal ODC activity

Table 2 summarizes the effects of TPA on epidermal ODC activity.

IV. Discussion

Although experience of Gerald Krueger, M.D., a Dermigen consultant, indicates that the hyperplasiogenic effects of skin wounding are localized to adjacent skin not more than 2 mm from the wound edge, the pilot experiment was designed to confirm that no such effects would be observed at the inter-biopsy distances to be employed in Protocol No. 910828. Comparison of sequential 3 mm biopsies taken at 6 and 24 hr post-final dosing with acetone provided no evidence of an effect of the first biopsy at 24 hr post-final dosing on the second biopsy taken 24 hr later, at least as judged by histopathologic assessment, measurement of epidermal thickness or labeling (BrdUrd) index. Similarly, comparison of sequential 4 mm biopsies taken at 6 and 24 hr post-final dosing with acetone provided no evidence of an effect of the first biopsy on the epidermal ODC activity of the subsequent biopsy. It is clear that wound-associated epidermal hyperplasia is very localized, and that the biopsy spacing proposed in Protocol No. 910828 is adequate to minimize effects at a distance.

The histology of tissue sections prepared from 3 mm skin biopsies was excellent and adequate for the evaluation of test article-induced effects on epidermal thickness and labeling index.

The homogenate from 3 pooled 4 mm skin biopsies taken 24 hr post-final dosing with TPA contained sufficient ODC activity to perform 3 separate triplicate assays. This indicates that a single 4 mm skin biopsy will provide enough material for a single triplicate assay, provided the ODC activity is $> 0.135 \text{ nmol CO}_2/\text{hr}/\text{mm}^2 (\times 10^3)$.

V. Recommendations

1. To optimize the measurement of ODC activity in biopsies from human skin xenografts, single 4 mm biopsies should be placed in 0.5 mL buffer, minced and homogenized using the small probe of the Polytron homogenizer. This should provide sufficient sample to assay ODC activity in triplicate 100 μL aliquots of the 30,000 xg supernate and leave sufficient supernate for assay of its protein concentration.
2. Measurements of epidermal thickness and labeling (BrdUrd) index should utilize two tissue sections separated by approx. 200 μm of depth within the same paraffin block. This will assure a sufficient linear length of epidermis to

meet the measurement requirements specified in Protocol No. 910828.

References

1. O'Brien, T.G. and Diamond, L. Ornithine decarboxylase induction and DNA synthesis in hamster embryo cell cultures treated with tumor-promoting phorbol diesters. *Cancer Res.*, 37: 3895-3900, 1977.
2. Kruszewski, F.H. and DiGiovanni, J. Alternations in epidermal polyamine levels and DNA synthesis following topical treatment with chrysarobin in SENCAR mice. *Cancer Res.*, 48: 6390-6395, 1988.
3. Aldaz, C.M., Conti, C.J., Gimenez, I.B., Slaga, T.J., and Klein-Szanto, A.J.P. Cutaneous changes during prolonged TPA applications and residual effects after cessation of treatment. *Cancer Res.*, 45: 2753-2758, 1985.

Table 1. Effect of TPA on Epidermal Hyperplasia.

Treatment	Epidermal thickness μm at hrs post- final dosing		Labeling (BrdUrd) index (%) at 48 hr post- final dosing
	24	48	
Acetone	14.2	N.D. ¹	N.D. ¹
	11.5	13.1	1.4
	14.2	11.5	2.3
TPA, 6.8 nmol	67.5	66.9	21.2
	41.3	38.5	12.9
	53.2	35.3	N.D. ²

¹Not determined. This animal died while under anesthesia for collection of the biopsy at 24 hr post-final dosing.

²Not determined due to complete absence of labeling.

Table 2. Effect of TPA on epidermal ODC activity

Treatment group	Hr post-final dosing	ODC Activity	
		nmole CO ₂ per hr/mg protein	nmole CO ₂ per hr/mm ² (x10 ³)
Acetone	6	<0 ^a	<0 ^a
	24	<0 ^a	<0 ^a
TPA, 6.8 nmole	6	1.36	2.36
	24	<0 ^a	<0 ^a

^aValues <0 indicate that the activity was less than that of the buffer control; i.e., no significant activity.